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**Original Article**

# Molecular Characterization of *Fasciola* spp. from a Donkey (*Equus asinus*) Using Partial Sequencing of *cox1* and *nad1*

**Mohammad ASADPOUR [1], *Hassan SHARIFIYAZDI [2], Mohammad MOAZENI [1], Seyed Hossein MALEKPOUR [1]**

1. *Department of Pathobiology, School of Veterinary Medicine, Shiraz University, Shiraz, Iran*
2. *Department of Clinical Sciences, School of Veterinary Medicine, Shiraz University, Shiraz, Iran*

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***Correspondence**
**Email:**
sharifiy@shirazu.ac.ir

***Abstract***

***Background:*** *Fasciola hepatica* as an important parasite affects health of humans and animals in some tropical and subtropical areas of the world, including Iran. Little is known about the molecular diversity of *Fasciola* in Equidae. Therefore, this study aimed to characterize the genetic polymorphisms among parasites.

***Methods:*** Eight adult *Fasciola* spp. isolates were collected from a working donkey after necropsy in Shiraz, southwestern Iran, in 2018. Primarily, various parameters were measured morphologically. Subsequently, DNA was extracted from each fluke and molecular markers of cytochrome C oxidase (*cox*1) and NADH dehydrogenase 1(*nad*1) from individual *Fasciola* isolates were amplified using PCR assay and sequence data were employed for molecular and phylogenetic analysis. Genetic diversity between isolates was evaluated by comparing the sequences of these two mitochondrial regions.

***Results:*** Based on the morphological and analyzed mitochondrial sequences, all of eight donkey isolates (100%) were identified as *F. hepatica*. Moreover, nine and five nucleotide polymorphisms were identified in the *cox*1and *nad*1 region sequences, respectively.

***Conclusion:*** Accordingly, phylogenetic data revealed five and four haplotypes among donkey isolates based on the *cox*1and *nad*1 markers. Similarly, some of these haplotypes have been previously reported from different host species in Iran as well as all around the world.

## Introduction

*Fasciola hepatica* is a zoonotic helminth parasite, with a considerable economic impact and public health concern all around the world (1). Fasciolosis caused by *F. hepatica* is a widespread helminth infection among herbivorous animals. Amphibious lymnaeid snails that serve as the intermediate hosts, release the cercariae, which quickly develop to metacercariae, the infective stage of the parasite for the final hosts. The animals become infected by eating the metacercariae through contaminated forage or drinking water (2).

Equines have vital importance in the subsistence of developing countries, particularly in Africa, especially for transportation (3). Horses are frequently resistant to infection with *Fasciola* (4,5). Conversely, others imply the high prevalence of fasciolosis in donkeys and mules (3, 6). Therefore, donkeys may play a remarkable role in the epidemiology of fasciolosis in both livestock and humans (6).

Even though equine fascioliasis may be diagnosed by fecal examination (7) or during postmortem studies (6). In many cases, fluke infections do not reach maturity in horses and eggs may not be seen in fecal examinations (8). Hence, serological and molecular methods have recently been taken into consideration for diagnosis of equine fasciolosis (9, 10). The aim of the present study was molecular characterization of *Fasciola* spp. obtained from one donkey, based on the markers of cytochrome C oxidase (*cox*1) and NADH dehydrogenase 1(*nad*1) in Iran, and assessment of their relationship with host specificity.

## Materials and Methods

### *Parasites*

Eight *Fasciola* samples were collected from the bile ducts of a naturally infected donkey, located at Shiraz, the capital city of Fars Province, Iran in 2018. After collection, the worms were washed 3 times with normal saline followed by fixation in 70% ethanol.

### *Morphometric measurements*

To determine the body size and differences, various parameters were measured using image analysis software (Digimizer® Version 4.6.1). Therefore, the body length, body width (BW), body area and body perimeter of flukes were measured individually. Additionally, cone length, cone width, distance between oral sucker and ventral sucker (OS–VS) and BL/BW ratio were also considered. Besides, the obtained morphometric results from these adult flukes were compared with previously published data on those provided from other host animals and human. All data obtained in this section are shown as Mean± Standard deviation (11-13).

### *DNA extraction*

All eight adult flukes were placed in Petri dishes individually, until complete alcohol evaporation. Then a piece from anterior part of each fluke was cut and transferred to a 1.5 ml microtube. DNA was extracted via a commercial extraction kit (DNeasy Blood & Tissue Kit®, Qiagen, Germany) according to manufacturer's procedure, but after adding the lysis buffer and proteinase K the samples were placed in a 56 °C water bath, overnight. Extracted DNA was kept at -20 °C until further use.

### *Amplification of cox1 and nad1 genes*

Both *cox*1and *nad*1 genes were amplified using two pairs of primer as follow; Ita 8 (F: 5'-ACG TTG GAT CAT AAG CGT GT-3') and Ita 9 (R: 5'-CCT CAT CCA ACA TAA CCT CT-3') for *cox*1 amplification (493 bp) (14) and Ita 10 (F: 5'-AGA TTC GTA AGG GGC CTA ATA-3') and Ita 2 (R: 5'-ACC ACT AAC TAA CTA ATT CAC TTT C-3') for *nad*1 amplification (534 bp) (15) were used as de-

scribed and used previously. We used a ready-to-use master mix (Taq DNA Polymerase Master Mix RED, Ampliqon, Denmark). For *cox*1 amplification, each 50 µl PCR tube was contained 25 µl of master mix supplemented with 8 µl DNA (~ 40 ng) and 2 µl (20 pmol) of each forward and reverse primer (Ita 8 and Ita 9) that reached to 50 µl with distilled water. Polymerase chain reaction (PCR) program for amplification of *nad*1 gene was performed using Bio-Rad PCR systems (Applied Biosystems, California, USA) as follow; PCR cycles were started with an initial denaturing step at 95 °C for 5 min followed by 35 cycles at 94 °C for 60 sec, 50.6 C for 45 sec and 72 °C for 45 sec with a final extension at 72 °C for 7 minutes. The *nad*1region was amplified using similar PCR conditions except annealing temperature, which was 58 °C for 45 sec and *nad*1specific primers (Ita 10 and Ita 2) were used.

### *Sequencing and nucleotide data analysis*

To characterize the molecular differences between all specimens, PCR products of *nad*1 and *cox*1 were sequenced directly using a capillary DNA analyzer (ABI 3730; Applied Biosystems, Foster City, California, USA). Nucleotide sequence analysis was undertaken using the National Center for Biotechnology information BLAST (Basic Local Alignment Search Tool) programs and databases. DNA sequences of closely related species were also downloaded and used in the phylogenetic analysis. Multiple sequence alignments were made with the Clustal W in MEGA 7.0 using default parameters. Molecular Phylogenetic analysis was conducted in MEGA 7.0 software (16). In phylogenetic analysis, the lengths of the *cox*1 and *nad*1 sequences were 493 and 534 nucleotides, respectively.

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. Support values for internal nodes were estimated using a bootstrap resampling procedure with 1000 replicates. Initial tree for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (16) approach and then selecting the topology with superior log likelihood value. The tree was constructed to scale, with branch lengths measured in the number of substitutions per site. All positions containing gaps and missing data were eliminated (9). In addition, *F. gigantica* accession nos. GQ398050 and KX036357 were used as out group taxa for *cox*1 and *nad*1 phylogenetic analyses, respectively.

### *Ethical Standards*

This study complied with the Ethical Principles in Animal Research, approved and performed following the ethical standards of the Committee for Animal Experiments of School of Veterinary Medicine, Shiraz University. The research was conducted under the permission of a farm owner who gave informed consent for the study to be carried out.

## Results

### *Morphometric evidence*

Morphological results of isolated adult flukes are presented in Table 1. Morphometric evaluation of parasite samples initially revealed that all isolates were *F. hepatica*. Figure 1 presents macrophoto of *Fasciola* sp. worms isolated in this study. Moreover, a comparison between the present morphometric results in adult *F. hepatica* and those previously reported from the different host origins in Iran, are presented in Table 2.

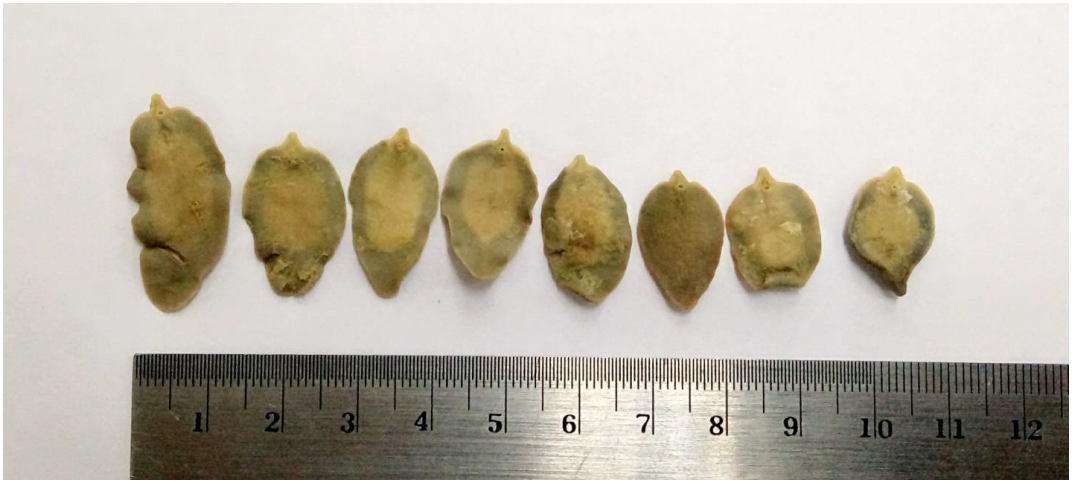


**Fig. 1:** Macrophoto of adult *F. hepatica* flukes isolated from naturally infected donkey in Shiraz, southwestern Iran

### *PCR and phylogenetic analysis results*

As expected, PCR products of *cox*1 (493 bp fragment) and *nad*1 (534 bp fragment) genes of all eight flukes' samples were amplified and successfully sequenced. The GenBank accession numbers were provided for *cox*1 (MF537583 to MF537590) and *nad*1 (MF628261 to MF628268) and for the flukes isolated from donkey in this study (Table 3).

Phylogenetic analysis of obtained sequences showed that all isolates had sequences identical to *F. hepatica*. The phylogenetic positions of isolates were placed in a broad frame and also compared with narrowly related parasites with different hosts and geographical regions that exist in the GenBank database. Partial *cox*1 sequence was found to consist of five polymorphic sites (17, 207, 219, 273 and 339) with at least four different haplotypes (A to D) (Fig. 2 and Table 4).

**Table 1:** Morphometrical data on *F. hepatica* adults isolated from a naturally infected donkey in Shiraz, southwestern Iran

| *Sample number* | *Body length (BL)* | *Body width (BW)* | *BL/BW ratio* | *Cone length* | *Cone width* | *Distance *OS–VS* | *Body area (mm²)* | *Body (Perimeter* |
|---|---|---|---|---|---|---|---|---|
| F1 | 3.28 | 1.56 | 2.102 | 2.51 | 2.83 | 2.04 | 3.647 | 9.629 |
| F2 | 2.49 | 1.54 | 1.60 | 2.24 | 2.94 | 2.13 | 2.670 | 7.549 |
| F3 | 2.57 | 1.31 | 1.96 | 1.87 | 2.54 | 2.17 | 2.364 | 7.535 |
| F4 | 2.53 | 1.39 | 1.82 | 1.97 | 1.85 | 2.42 | 2.425 | 7.722 |
| F5 | 2.34 | 1.31 | 1.78 | 1.53 | 2.08 | 1.91 | 2.238 | 6.851 |
| F6 | 2.17 | 1.28 | 1.70 | 1.68 | 2.58 | 1.82 | 1.944 | 6.623 |
| F7 | 2.22 | 1.43 | 1.55 | 2.21 | 3.11 | 2.36 | 1.949 | 7.189 |
| F8 | 2.01 | 1.34 | 1.50 | 1.92 | 2.88 | 2.39 | 1.663 | 8.492 |
| Mean±SD | 2.451±0.38 | 1.395±0.10 | 1.751±0.20 | 1.99±0.31 | 2.60±0.43 | 2.15±0.22 | 236.2±0.60 | 76.98±0.96 |

* Distance between the oral sucker and the ventral sucker (OS-VS)

**Table 2:** Morphometric data of adult *Fasciola hepatica* form different host origins in Iran

| *Species* | *Body length* | *Body width (BW)* | *BL/BW ratio* | *Cone length* | *Cone width* | *Distance *OS–VS* | *Body area ($mm^2$)* | *Body Perimeter (mm)* | *Reference* |
|---|---|---|---|---|---|---|---|---|---|
| Donkey | 2.451±0.38 | 1.395±0.10 | 1.751±0.20 | 1.99±0.31 | 2.60±0.43 | 2.15±0.22 | 236.2±0.60 | 76.98±0.96 | Present study |
| Cattle | 2.975±0.61 | 1.227±0.15 | 2.777±0.57 | ND | ND | ND | 333.9±0.70 | 76.26±0.92 | 14 |
| Cattle | 4.930±1.17 | 1.140±0.20 | 4.400±1.08 | ND | ND | ND | 515.3±1.88 | 111.70±2.86 | 21 |
| Cattle | 2.308 ± 5.27 | 9.93 ± 1.79 | 2.32 ± 0.33 | 2.26 ± 0.36 | 2.92 ± 0.41 | 1.73 ± 0.38 | 235.2 ± 86.6 | ND | 29 |
| Sheep | 2.11±0.2 | 1.2±0.15 | 1.46±2.09 | 0.41±0.07 | 0.23± 0.05 | 0.28±0.08 | ND | ND | 30 |
| Sheep | 2.817 ± 5.14 | 11.33 ± 1.47 | 2.43 ± 0.49 | 2.47 ± 0.43 | 3.13 ± 0.62 | 1.97 ± 0.48 | 322.5 ± 83.96 | ND | 29 |
| Sheep | 2.146±5.06 | 0.948±2.74 | 2.380±0.67 | 2.400±0.69 | 3.200±0.66 | 1.350±0.44 | 161.1±7.50 | ND | 28 |
| Goat | 2.731 ± 3.67 | 1.18 ± 2.49 | 2.37 ± 0.43 | 2.26 ± 0.38 | 2.91 ± 0.41 | 1.81 ± 0.31 | 322.5 ± 63.36 | ND | 29 |
| buffalo | 4.270±0.77 | 0.990±0.42 | 4.340±0.97 | ND | ND | ND | 415.5±1.64 | 99.25±1.63 | 21 |
| buffalo | 3.00±1.62 | 1.40±0.87 | 2.142±1.82 | ND | ND | 0.50±0.23 | ND | ND | 32 |
| Buffalo | 4.07±0.49 | 2.3±0.23 | 1.67±2.43 | 0.67±0.03 | 1.04±0.57 | 0.63±0.03 | ND | ND | 30 |
| Human | 2.30 | 9 | 2.55 | ND | ND | ND | 122 | 43 | 31 |
| Human | 1.90 | 7.50 | 2.53 | ND | ND | ND | 162 | 52.70 | 31 |

ND=no data

**Table 3:** Haplotyes and accession numbers based on the *cox1* and *nad1* for *F. hepatica* adults isolated from a naturally infected donkey in Shiraz, southwestern Iran

| *Sample number* | *cox1 haplotype* | *cox1 accession no.* | *nad1 haplotype* | *accession no* |
|---|---|---|---|---|
| F1 | D | MF537583 | D | MF628261 |
| F2 | B | MF537584 | B | MF628262 |
| F3 | A | MF537585 | A | MF628263 |
| F4 | B | MF537586 | C | MF628264 |
| F5 | D | MF537587 | D | MF628265 |
| F6 | C | MF537588 | E | MF628266 |
| F7 | D | MF537589 | D | MF628267 |
| F8 | C | MF537590 | D | MF628268 |

**Table 4:** Intraspecific variation in the *cox*1 sequences of F. *hepatica* from donkey in Shiraz, southwestern Iran

| *Polymorphic positions* | *cox1 haplotypes in Donkey* | | | |
|---|---|---|---|---|
| | A (n=1) | B (n=2) | C (n=2) | D (n=3) |
| 17 | T | T | T | G |
| 207 | C | C | T | T |
| 219 | G | T | T | T |
| 273 | G | T | T | T |
| 339 | G | A | A | A |

In comparison with partial *cox*1 sequence, partial sequence of *nad*1 showed more intraspecific variation among our eight isolates (A to E) (Fig. 3 and Table 5). Based on the analyzed *nad*1 sequence data, at least nine polymorphic positions and five haplotypes were identified as polymorphic denoting perceptible nucleotide diversity among isolates of the parasite in the current study (Table 5). Variation in nucleotide sequences among the eight isolates ranged from 0% to 0.95% for *cox*1 and 0% to 1.12% for *nad*1.

**Table 5:** Intraspecific variation in the *nad*1 sequences of *Fasciola hepatica* from donkey in Shiraz, southwestern Iran

| ***Polymorphic positions*** | ***nad1 Genotypes in Donkey*** | | | | |
|---|---|---|---|---|---|
| | A (n=1) | B (n=1) | C (n=1) | D (n=4) | E (n=1) |
| 13 | C | C | C | T | T |
| 29 | A | G | G | G | G |
| 60 | A | G | G | G | G |
| 71 | A | G | G | A | A |
| 246 | C | T | T | T | T |
| 296 | T | T | T | C | C |
| 309 | A | G | A | A | A |
| 352 | C | C | C | C | T |
| 327 | T | C | T | T | T |

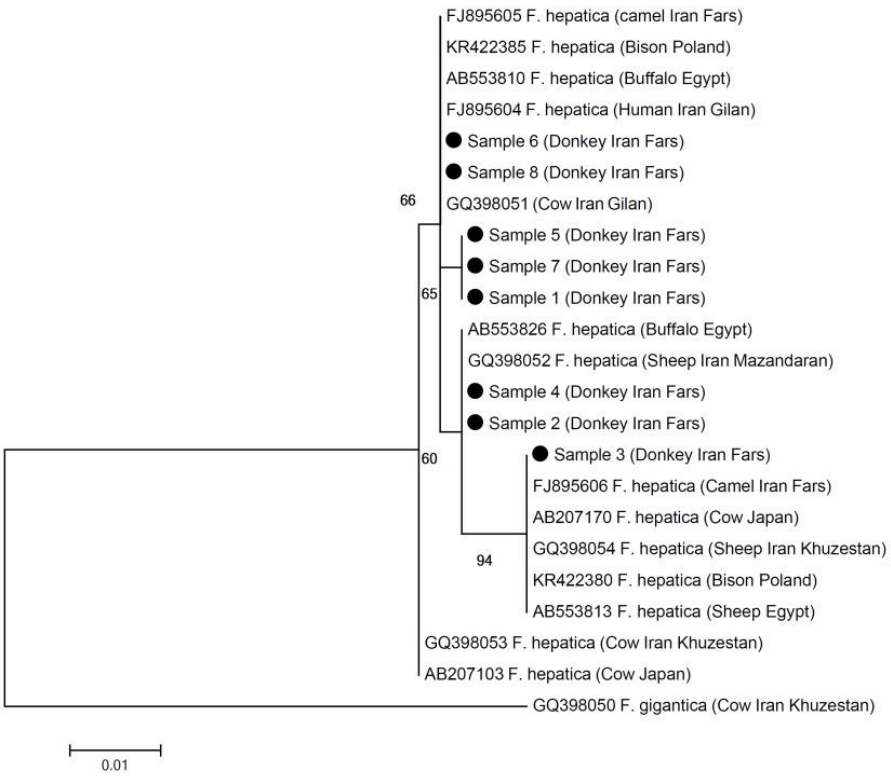


**Fig. 2:** Phylogenetic tree based on partial *cox*1 sequences, constructed according to the Maximum Likelihood method, showing the position of Iranian donkey *Fasciola* isolates (black dots)

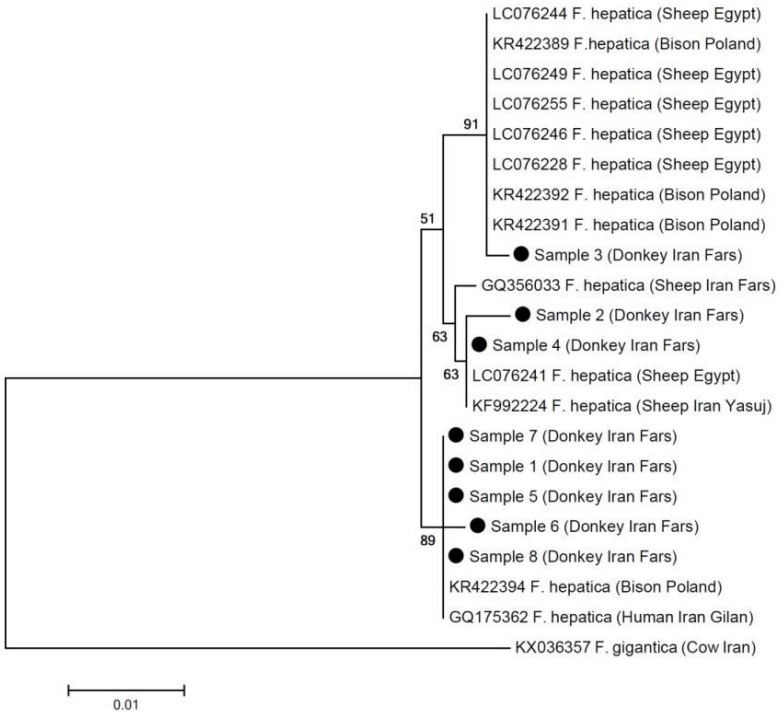


**Fig. 3:** Phylogenetic tree based on partial *nad*1 sequences, constructed according to the Maximum Likelihood method, showing the position of Iranian donkey *Fasciola* isolates (black dots)

## Discussion

Members of the genus *Fasciola* (Platyhelminthes: Digenea) are responsible for many important helminth infections with socioeconomic impacts, worldwide (1, 2). Fasciolosis predominantly caused by *F. hepatica* is quite common disease in humans and a wide range of domesticated animals (1, 10). Next to domestic ruminants; donkeys can play a significant role in the epidemiology of both livestock and human fasciolosis (17-19). Due to limitations of morphological methods, many molecular approaches have been designed and used for identification and genetic differentiation of fasciolides (14, 15). In this regard, numerous molecular studies have been devoted to phylogenetic analysis of *F. hepatica* in various hosts and geographical locations (15, 20, 21), but a very small portion of those researches have been assigned on equines isolates (22). Nowadays, *cox*1 and *nad*1 as two mitochondrial markers are widely used for genetic differentiation of parasites (14, 15, 21, 23). Therefore, these two polymorphic *mt*DNA genes were used in this study to clarify the genetic diversity within *Fasciola* isolates collected from the bile ducts of a donkey in Iran. Moreover, morphometric parameters as helpful criteria for differentiation between *F. hepatica* and *F. gigantica* (24, 25) were also considered in this work. As shown in Table 1, morphometric measurements in the current study revealed that BL/BW values of all the eight flukes were in the range of that for *F. hepatica*. Some researchers reported the BL/BW ratio nearly 2 in *F. hepatica* and up to 3 in *F. gigantica*, whereas the accurate ranges of mentioned values are 1.29-2.80 and 3.40-6.78 for *F. hepatica* and *F. gigantica*, respectively (26). However, similarity in morphometric size among our isolates is likely due to the limitation in samples source.

The literature offers contradictory data on the size of liver flukes from different animals, which are more remarkable in BL and BW versus other morphometric parameters. Mor-

phometric values in BL and BW areas in current study were mostly in the same range of previously reported sheep and goat isolates (11, 27, 28) (Table 5). Conversely, these values were smaller in buffalo, cattle, and also human isolates (21, 28-30). Although morphometric values of adult flukes from different host populations (sheep, cattle, goat, buffalo, human and donkey) showed great overlap in some measurements, there were clear allometric differences in certain morphological traits that characterize the *F. hepatica* in different host origins (31). Likewise, an allometric study revealed that the final host species decisively influences the size of the adult flukes even within the same geographical locations (32).

Our morphological findings were compatible with molecular results and parasite samples had sequences identical to *F. hepatica*. Based on analyzed sequences data, genetic polymorphism among *Fasciola* isolates for *nad*1 (nine polymorphic positions) was higher versus *cox*1 (five polymorphic positions) gene. Accordingly, molecular typing based on *cox*1 and *nad*1 sequences data has generated four and three different haplotypes, respectively. Importantly, some of these identified haplotypes were reported previously from different hosts in Iran as well as in other parts of the world (15, 29). For instance, haplotype *cox*1-A has been isolated from camel (AN: FJ895606) and sheep (AN: GQ398054) in Iran, cow in Uruguay (AN: AB207170), sheep in Egypt (AN: AB553813) and Bison in Poland (AN: KR422380). Moreover, no association was observed between geographic area and phylogenetic ancestry in this study, and this finding is in agreement with previous investigation, which indicated that host and geographical associations are not suitable factors for classification of *Fasciola sp.* (15). In addition, accurate identification of haplotype may be impossible by using a host.

In a molecular study conducted on the 130 isolated *Fasciola* collected from abattoirs in 9 provinces of Iran, 37 *nad1* haplotypes were detected (32). In another study, partial sequences of *mt*DNA showed eight haplotypes based on *cox1* and *nad1* genes among 90 *F. hepatica* collected from 30 cattle at slaughterhouse located in three different geographical locations in the North-East of Iran (33). *F. hepatica*, lives in Eastern provinces of Iran, had different genetic structures compared with the other *Fasciola* populations in the world (34). Similarly, the sequences obtained from north and southwest isolates had substantial differences mainly in *cox1* region (35). However, Moazeni et al. suggested that limitation in geographical sampling and number of isolates present bias into phylogenetic analysis (14).

To the best of our knowledge, the current study is the first molecular typing of *F. hepatica* in donkeys in Iran. Based on the data obtained in this work, range of nucleotide difference among our isolates using *cox*1 (0% to 0.95%) and *nad*1 (0% to 1.12%) was limited. Similarly, evidence showed limited nucleotide diversity on *cox*1 sequence (0% to 0.98%) among cattle and sheep isolates of *F. hepatica* in various regions of Iran (14). However, genetic variation among Iranian isolates of *F. gigantica* showed a broader range (0% to 4.63%) (21). Likewise, in a previous molecular study from Tunisia, which conducted on horse isolates using a ribosomal marker (*ITS* rDNA sequence), no genetic diversity detected and all *Fasciola* isolates were identical to those previously reported for *F. hepatica* (22).

## Conclusion

All of eight flukes isolated from donkey in this work were identified as *F. hepatica* with significant genetic polymorphisms at both *cox*1and *nad*1 mitochondrial regions. Moreover, no remarkable relationships were found between animal sources and phylogenetic lineage. This genetic variation likely is due to differences in virulence, animal sources and drug susceptibility/resistance (14, 23, 36). Finally, more studies are recommended to clarify the role of donkey on the epidemiology of *F. hepatica.*

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## Conflict of interest

The authors declare that they have no conflict of interest.

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